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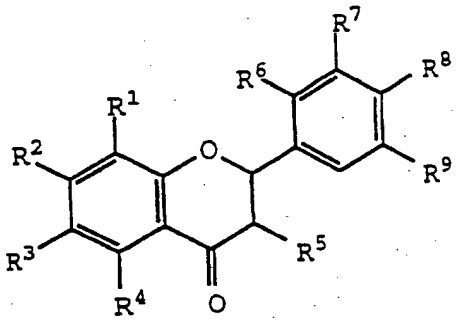
Remarks:
This application was filed on 09 - 03 - 1996 as a divisional application to the application mentioned under INID code 62.

(54) Chondroprotective flavonoids

(57) The invention relates to the use of a flavonoid compound of the general formula (I):



(I)

wherein R^1 to R^9 are, independently, a hydrogen atom, hydroxyl group or methoxyl group or a stereoisomer thereof, or a naturally occurring glycoside thereof.

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Description

The invention relates to the use of an agent for protecting cartilage, i.e., a chondroprotective agent, more particularly, a chondroprotective agent containing a flavonoid compound according to formula I or a stereoisomer thereof, or a naturally occurring glycoside thereof.

There are various types of arthropathy, for example, rheumatoid arthritis, rheumatic fever, and osteoarthritis. Many people particularly suffer from rheumatoid arthritis and osteoarthritis. These diseases have been studied as the major types of arthropathy. There are congenital and secondary osteoarthritis, and further primary osteoarthritis caused by degeneration of the articular cartilage along with aging. Patients suffering from primary osteoarthritis have recently been increasing along with the increase in the population of the aged.

Although there are considerable differences of the causes and conditions between rheumatoid arthritis and osteoarthritis, the articular function becomes eventually obstructed by the destruction of the cartilage in both of rheumatoid arthritis and osteoarthritis.

The first choice of medicines for the treatment of rheumatic diseases such as rheumatoid arthritis, rheumatic fever, systemic lupus erythematosus, and ostecarthritis are analgesic and anti-inflammatory agents, for example, aspirin or indometacin. Further, gold compounds such as Shiosol, immunomodulators, steroids, or D-penicillamine are used as medicines for the treatment of rheumatoid arthritis.

The above conventional analgesic and anti-inflammatory agents, however, were not effective against the destruction of the articular cartilage, and in fact, sometimes exhibited adverse effect in experiments using chondrocytes. Further, no inhibitory effect on articular cartilage destruction was also observed in the above-mentioned medicines for the treatment of rheumatoid arthritis.

It is known that flavonoids may be used as an agent for protecting a blood vessel and further in the following pharmaceutical applications: a virus genome deactivating agent for apigenin, chrysin, morin, fisetin, and baicalein [Japanese Unexamined Patent Publication (Kokai) No. 2-101013], an agent for determining the function of polymorphonuclear leukocyte for flavonoids [Japanese Unexamined Patent Publication (Kokai) No. 63-253254], an oral agent for suppressing smoking for flavonoids [Japanese Unexamined Patent Publication (Kokai) No. 4-46119], treatment of high protein edema for rutin, diosmin, and the like (U.S. Patent No. 5,096,887), an anti-tumor agent containing flavonoids [Japanese Unexamined Patent Publication (Kokai) No. 3-275625], an anti-tumor agent containing apigenin [Japanese Examined Patent Publication (Kokoku) No. 3-61644], an agent for suppressing the formation of peroxylipid for hesperetin, kaempferol, and the like [Japanese Unexamined Patent Publication (Kokai) No. 3-54231], an anti-tumor agent containing kaempferol (Japanese Unexamined Patent Publications (Kokai) No. 4-103529 and No. 4-103532], a calcium antagonist for hesperidin and luteolin [Japanese Unexamined Patent Publication (Kokai) No. 4-243822], a sialidase inhibitor for luteolin [Japanese Unexamined Patent Publication (Kokai) No. 64-42427], an anti-retrovirus agent for luteolin [Japanese Unexamined Patent Publication (Kokai) No. 3-7224], an anti-HBV (hepatitis B virus) agent for quercetin [Japanese Unexamined Patent Publication (Kokai) No. 4-234320], and the like.

Flavonoids have not, however, been known to be useful as chondroprotective agents.

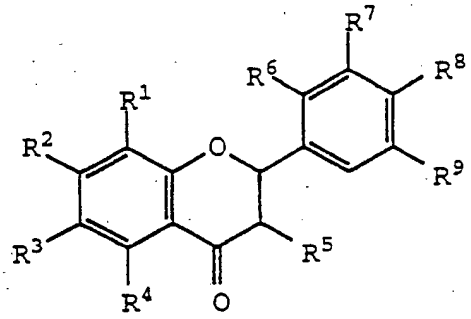
Agents and Actions, vol. 12, 3 (1982) p. 298, describes the inhibiting action of nepitrin on inflammation in adjuvant athritis in a comparable level to phenylbutazone treatment. Z. Rheumatol, vol. 42 (1983) p. 203-205 refers to the inhibition of arthritis by rotusid containing medicaments. Chem. Pharm. Bullet., vol. 32, p. 2724-2729, discloses that baicalin, baicalein and wogonin show bone degeneration inhibiting effects in adjuvant-induced arthritis. However, bone degeneration is the degradation of the type I collagen.

US-A-4 268 517 discloses a pharmaceutical composition for treating a generative disease of the articular cartilage containing (+)-catechin. Arch. Pharm. Res., vol. 16(1) 1993) p. 25-28, refers to antiinflammatory activity of naturally occuring flavone and flavonol glycosides. Fitoterapia, 1990, vol 90(5), p. 460-461, discloses the anti-inflammatory activities of various flavonoid compounds.

Accordingly, the object of the present invention is to provide a chondroprotective agent containing as an active ingredient a particular flavonoid compound or a stereoisomer thereof, or a naturally occurring known glycoside thereof.

Other objects and effects of the present invention will be clear from the following description.

The present invention relates to the use of a flavonoid compound of the general formula I:



(I)

wherein R^1 to R^9 are, independently, a hydrogen atom, hydroxyl group, or methoxyl group, or a stereoisomer thereof, or a naturally occurring glycoside thereof (hereinafter referred to as "the present substance") for the preparation of a chondroprotective agent.

According to the invention a chondroprotective agent is provided for suppressing the destruction of the articular cartilage and it was found that the particular flavonoid compounds and stereoisomers thereof, and the naturally occurring known glycoside thereof showed significant inhibition of the depletion of proteoglycan which is a major component of the cartilage matrix, and therefore, are useful as a chondroprotective agent for prohibiting the destruction of the articular cartilage.

DETAILED DESCRIPTION OF THE PREFERRED EMBODYMENTS

The active ingredient of the chondroprotective agent according to the present invention is a flavonoid, which is widely present in the vegetable kingdom. Typical flavonoid compounds include flanvanones and flavanonols. Flavanones contain an asymmetric carbon atom at the 2-position, and flavanonols contain asymmetric carbon atoms at the 2- and 3-positions, and such compounds may be present as the stereoisomers. These stereoisomers can also be used in the present invention. Further, the saccharides present in the above naturally occurring glycosides are not particularly limited. As examples of the naturally occurring glycosides, there may be mentioned glucoside, galactoside, fructoside, rhamnoside, rutinoside (that is rhamnoglucoside), arabinoside, xyloside, apioglucoside, and robinobioside.

In the present invention, any naturally occurring flavonoids may be used as the above present substance. The flavonoid compounds and naturally occurring glycosides thereof shown in the following Table 1 are preferable.

Table 1

No.	Name	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	R^9	x
1	Pinocembrin	H	OH	H	OH	H	H	H	H	H	Single
2	Naringenin	H	OH	H	OH	H	H	H	OH	H	ditto
3	Salipurpin	H	OH	H	0Glu	H	H	H	OH	H	ditto
4	Prunin	O	OGlu	H	OH	H	H	H	OH	H	ditto
5	Naringin	H	ORha	H	OH	H	H	H	OH	H	ditto
6	Sakuranetin	H	$0CH_3$	H	OH	H	H	H	OH	H	ditto
7	Sakuranin	H	$0CH_3$	H	OGlu	H	H	H	OH	H	ditto
8	Hesperetin	H	OH	H	OH	H	H	OH	OCH_3	H	ditto
9	Hesperidin	H	ORut	H	0H	H	H	OH	OCH_3	H	ditto
10	Eriodictyol	H	OH	H	OH	H	H	OH	OH	H	ditto
11	Eriodictin	H	ORha	H	OH	H	H	OH	OH	H	ditto
12	Pinobanksin	H	OH	H	OH	OH	H	H	H	H	ditto
13	Aromadendrin	H	OH	H	OH	OH	H	H	OH	H	ditto
14	Engelitin	H	OH	H	OH	ORha	H	H	OH	H	ditto
15	Fustin	H	OH	OH	OH	H	H	H	H	H	ditto
16	Taxifolin	H	OH	H	OH	OH	H	OH	OH	H	ditto
17	Astilbin	H	OH	H	OH	0Rha	H	OH	OH	H	ditto
18	Ampelopsin	H	OH	H	OH	0H	H	OH	OH	OH	ditto

0Glu: Glucoside, 0Apg: Apioglucoside, ORut: Rutinoside,
OGal: Galactoside, ORha: Rhamnoside, ORob: Robinobioside

The compounds in Table 1 include a single bond as X. Thus, the carbon atom in the 2-position or the carbon atoms in the 2- and 3-positions are asymmetrical, and there exist stereoisomers. It is known that pinocembrin includes (±) and (S) isomers; naringenin, sakuranetin, hesperetin and eriodictyol include (±), (R), and (S) isomers; pinobanksin includes (2R-trans) and (2S-trans) isomers; aromadendrin and fustin include trans-(±), (2R-trans), and (2S-trans) isomers; taxifolin and ampelopsin include trans-(±), (2R-trans), (2S-trans), and (2R-cis) isomers.

It is possible to use, as the flavonoids, compounds isolated and purified from naturally occurring plants or chemically synthesized. Many compounds described in Table 1 are commercially available. For example, it is possible to obtain hesperetin and hesperidin from Funakoshi Co., Ltd., Tokyo.

No abnormalities were observed for a week after acacetin was administered orally-to BALB/c mice (female, seven weeks old) at the dose of 100 mg/kg. The same results were obtained where hesperidin was administered.

As a pharmacological effect, the present substance exhibits the function to inhibit destruction of chondrocyte matrix in chondrocyte culture (derived from cartilage of rabbit shoulder and knee joints) (see Example 1 as below).

Accordingly, the present substance is useful as a chondroprotective agent for treating various types of arthropathy accompanying the cartilage destruction of joints. Examples of such arthropathy are rheumatoid arthritis, osteoarthritis, periarthritis humeroscapularis, shoulder-armneck syndrome and lumbago.

The chondroprotective agent containing the present substance as an active ingredient may be in the form of any conventional formulation. The chondroprotective agent may contain the present substance alone, or a mixture of the present substance with any pharmaceutically acceptable carrier or diluent. The chondroprotective agent may contain the active ingredient in an amount of 0.01 to 100 percent by weight, preferably 0.1 to 70 percent by weight.

The chondroprotective agent of the present invention may be administered orally or by some other routes.

The dose of the chondroprotective agent according to the present invention varies with the patient (animal or human), age, individual differences, state of illness, and the like. Generally speaking, however, when a human is treated, the dose of oral administration of the present substance is in the range of 0.1 to 500 mg/kg (body weight) per day, preferably

0.5 to 200 mg/kg (body weight), which is usually divided into 1 to 4 dosages in a day, although the dose outside the above range may sometimes be administered.

EXAMPLE

The present invention now will be further illustrated by, but is by no means limited to, the following Examples.

Example 1: Effect of Test Comyounds on Proteoalycan Depletion in Chondrocyte Culture

(a) Preparation of Cultured Chondrocytes

The cartilages were sterilely extracted from the shoulder and knee joints of rabbits (New Zealand White Rabbits) (body weight of 1 to 1.5 kg). The cartilages were thoroughly washed with PBS (-) (free of Ca^{2+}, Mg^{2+}), Hanks' solution and 0.1% EDTA-PBS (-), and then cut into small segments (1 mm x 1 mm x 1 mm). After PBS (-) containing 0.1% EDTA was added, the segments were allowed to stand in an incubator of 37°C for 30 minutes. Then, the segments were treated with a trypsin solution (0.25%) at 37°C for one hour to remove the connective tissue adhered to the cartilage. After the supernatant had been removed, the cartilages were treated for about 2 to 2.5 hours in a Ham F-12 medium containing 10% fetal bovine serum (FBS) and 0.2 % collagenase. Then, the collagenase solution was centrifuged (1500 r.p.m.), and the residual chondrocytes were washed twice with a Ham F-12 medium (chondrocyte culture medium) containing 10 % FBS. Finally, the resulting suspension was adjusted so that the chondrocytes were suspended in the concentration of 3×10^5 cells/ml in the chondrocyte culture medium. The chondrocytes were seeded in an amount of 1 ml/well on 24-well plates. The chondrocytes became confluent after 4 days. The experiment were performed within two weeks after reaching the confluent stage.

(b) Addition of Compounds to be tested and Proteoglycan Depleting Agents

The chondrocyte culture medium which had been used for cultivating the chondrocytes was removed from each well and 800 µl of fresh serum-free S-Clone medium containing 0.1% human serum albumin was added. Further, 100 µl of S-Clone medium containing the compounds to be tested (containing the compound in the concentration of 10 fold the final concentration; DMSO concentration = 2.5%) was added. The chondrocytes were cultured in the presence of carbon dioxide (5%) and air (95%) for 2 hours. Then, the proteoglycan depleting agent, PMA (phorbol myristate acetate) (final concentration = 0.1 µg/ml) was added into the culture medium of the chondrocytes.

The compounds to be tested were as follows: Compounds of present invention: hesperetin (present substance No. 8, (S) isomer), and hesperidin (present substance No. 9, (S) isomer) (all from Funakoshi Co.).

Comparative substance: Indometacin (Sigma Chemical Co.)

(c) Determination of proteoglycan

Proteoglycan depletion was determined by the measurement of the glycosaminoglycan (major constituent of proteoglycan, hereinafter referred to as GAG) content following digestion of the chondrocyte matrix with papain.

After 2 days, the supernatant of the chondrocyte culture was removed. Then, 1 ml of 0.03% papaine solution was added to the remaining chondrocyte matrix layer and a reaction was performed at 65°C for 1 hour to liberale the GAG from the matrix layer. The content of the GAG in the treated papaine solution was determined by the 1,9-dimethylmethylene blue method (refer to R.W. Farndale, Biochim. Biophys. Acta., Vol883, pp. 173 to 177, 1986). The GAG content in the chondrocyte matrix of the control test wherein the proteoglycan depleting agent was not added was shown as "100", and the relative amount of the GAG of each experiment except the control test was calculated by following formula:

$$\text{GAG relative amount (\%)} = (B/A) \times 100$$

wherein A represents the GAG content of the control tests wherein the proteoglycan depleting agent was not added, and B represents the GAG content wherein the proteoglycan depleting agents were added alone or the GAG content wherein the proteoglycan depleting agents and the compounds to be tested were added. The GAG contents of the control tests varied in a range of 11.23 to 59.0 µg/ml, depending on the period from the time when the chondrocytes became confluent until the time when the chondrocytes were used in the above experiment.

The results are shown in Table 2. The GAG content is the value of the mean value ± standard error (n = 3 to 6). For each of the compounds to be tested, the control test and the proteoglycan depleting test wherein the proteoglycan depleting agent was added were carried out and the results thereof are also shown. The significance was determined by Student's t-test with respect to the proteoglycan depleting test wherein the proteoglycan depleting agent was added. The results of the determination are shown as follows:

*: $P < 0.05$;
**: $P < 0.01$;
***: $P < 0.001$.

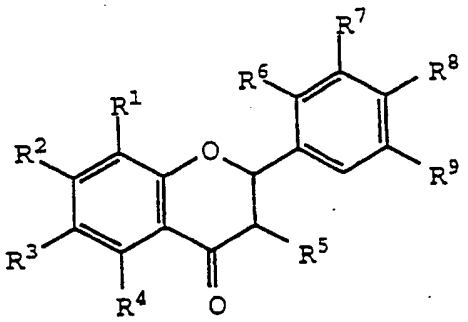
In comparison with the GAG content in the control tests wherein the proteoglycan depleting agent was not added, the addition of the proteoglycan depleting agents, PMA, induced a loss of GAG content. Under these conditions, the present compound significantly inhibited or reduced the loss of GAG content, and showed a function to inhibit or suppress the proteoglycan depletion. On the other hand, indometacin, a conventional analgesic and anti-inflammatory agent, did not show the function to inhibit or suppress the proteoglycan depletion, but caused a significant exacerbation on the proteoglycan depletion.

Table 2

Samples	GAG content (μg/ml)	(Relative amount of GAG) (%)
Control	56.1±0.8***	(100)
PMA	20.4±0.7	(36.4)
PMA + No. 9 (100 μm)	24.0±0.6	(42.8)
Control	59.0±0.9***	(100)
PMA	21.1±0.6	(35.8)
PMA + No. 8 (100 μm)	28.7±0.4***	(48.6)
Control	28.0±0.7***	(100)
PMA	15.4±0.5	(55.0)
PMA + indometacin		
(10 μM)	13.2±0.6*	(47.1)
(33 μM)	11.7±0.8**	(41.8)

Claims

1. Use of a flavonoid compound of the general formula (I):



(I)

wherein R^1 to R^9 are, independently, a hydrogen atom, hydroxyl group or methoxyl group or a stereoisomer thereof, or a naturally occurring glycoside thereof for the preparation of a chondroprotective agent.

2. Use according to claim 1, wherein said flavonoid compound is one or more compounds selected from the group consisting of pinocembrin, naringenin, salipurpin, prunin, naringin, sakuranetin, sakuranin, hesperetin, hesperidin, eriodictyol, eriodictin, pinobanksin, aromadendrin, engelitin, fustin, taxifolin, astilbin and ampelopsin.

3. Use according to claim 1 or 2, wherein said naturally occurring glycoside is one or more compounds selected from the group consisting of glucoside, galactoside, fuactoside, rhamnoside, rutinoside, arabinoside, xyloside, apioglucoside, and robinobioside.

4. Use according to any one of claims 1 to 3 wherein said flavonoid compound is hesperetin (S-form) and/or hesperidin (S-form).

5. Use according to any one of claims 1 to 4 wherein said flavonoid compound, stereoisomer thereof, or naturally occurring glycoside thereof is an extracted substance from a naturally occurring material.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 96 10 3715

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	Z. RHEUMATOL., vol. 42, page 203 G. WILHELMI ET AL. 'zur Prüfung potentieller Antiarthrotika an der spontanen Arthrose der Maus' *see the whole document *	1-3,5	A61K31/365 A61K31/70
Y	ARCH. PHARM. RES., vol. 16, no. 1, pages 25-28, S.J. LEE ET AL. 'antiinflammatory activity of naturally occurring flavone and flavonol glycosides' * see the whole document, especially Figure 1 and Table 1 *	1-5	
Y	US-A-4 268 517 (NIEBES ET AL.) 19 May 1981 * see the whole document*	1-5	
T	WO-A-94 14432 (DOLISOS LAB ;FRANCHIMONT PAUL (BE); BASSLEER CORINE (BE); ANGENOT) 7 July 1994 * see claim 1, page 2 line 16 - page 3 line 25 *	1-5	TECHNICAL FIELDS SEARCHED (Int.Cl.6) A61K

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	15 April 1996	Isert, B

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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